Investigation of Highest Yield of Prodigiosin Extracted from Serratia Marcescens Utilizing Multiple Natural Materials and Evaluation Pigments' Ability as an Antioxidant[1]

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ABSTRACT

Thirty samples were collected from environmental samples, they were taken from soil of different Mosul gardens to investigate the presence of *Serratia marcescens*. The initial diagnosis of *S.marcescens* was based on their ability to produce red pigment by culturing them on the nutrient agar medium**,** and complete the molecular diagnosis of the isolates by detecting the presence of the *16SrRNA* gene in our local isolates. Two isolates(S3 and S4) of *S. marcescens* bacteria were obtained from 30 soil samples. The two isolates were red pigment producing. The molecular of the diagnosis of the two bacterial isolates were obtained, and it was confirmed conclusively that they are *S. marcescens*, they gave very high similarity ratios 99.41 and 99.17% for S3 and S4 strain respectivly with the internationally registered *S. marcescens* bacteria. The highest yield of this pigment was investigated using Luria-Bertani(LB) media supplemented with available materials (sesame, peanut, coconut seeds and wheat bran). Sesame powder medium produced the highest prodigiosin production (493.396,423.607) unit/cell, followed by peanut medium (462.973, 406.660) unit/cell for two isolates S3 and S4 respectively. However, Wheat bran gave a lower concentration of pigment and coconut seeds failed to induce bacteria to synthesize the pigment. The antioxidant activity of the pigment was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the value was compared with the antioxidant activity of ascorbic acid.The results showed that the activity of the pigment in scavenging the free radicals of DPPH increased with increasing its concentration.

Keywords: *Serratia marcescens; Sesame; Peanut; Wheat bran; Coconut; Prodigiosin; DPPH.*

INTRODUCTION

Serratia marcescens(*S. marcescens*) is a motile, Gram-negative, rod-like bacteria that belongs to the Enterobacteriaceae family. It is a facultative anaerobic bacteria that can exist and live in different environments such as soil, water, plants and hospitals[1]. *S. marcescens* can be distinguished from other genera by its production of three special enzymes: DNase, Lipase and Gelatinase, and its production of a red pigment known as prodigiosin, because it has a group of genes that are associated with the prodigiosin operon[2].

Prodigiosin pigment is one of the secondary metabolite compounds and from the prodiginine family with a chemical composition consisting of three pyrrole rings. The molecular formula of prodigiosin is $C_{20}H_{25}N_3O$ [3]. Prodigiosin shows wide variety of biological properties including antibacterial, antifungal, anti-malarial, antioxidant, anti-proliferative, immunosuppressive and anticancer properties. In addition, prodigiosin has been applicated as a natural colorant of candles, textile materials, soap, paper, and ink[4-7].

Like other pigments[8], several studies have been conducted to determine the basic requirements for the growth of biomass of bacteria and to enhance their production, with interest in determining the best carbon source, nitrogen source, pH, temperature, fermentation time and mineral ions present during fermentation. The production of prodigiosin is still small-scale because the strategies used and reliance on commercial sources are expensive and complex. low-cost organic sources provide effective biological production that reduces the cost of production with a better content of prodigiosin compared to commercial sources [6,9,10], we resorted through experiments in reducing the costs of the vital processes

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which are involved in the biotechnological production of prodigiosin by using a group of low-cost natural materials as basic materials for the growth of *S. marcescens* bacteria and enhancing their production of prodigiosin.

MATERIALS AND METHODS

Isolation and diagnosis: A total 30 samples were collected from garden soils of different geographical regions of Mosul city/Iraq according to the method used by Othman *et al.* [11] , by transferring one gram of each soil sample into bottles of 50 ml capacity containing 10 ml of sterile peptone water medium at a concentration of 0.1% to make a suspension of soil, The bottles were kept at room temperature for 3 hours, after dilutions of the samples were made with peptone water to 10^{-7}, the dilutions 10^{-6} and 10^{-7} were cultured on nutrient agar medium and then incubated at 28°C for 2-3 days. After the end of the incubation period, the colonies were examined with red pigment,then were purified by growing them in the same media using the streaking method and incubated under the same conditions for 24 hours.

Molecular diagnosis

DNA extraction: DNA was extracted from two bacterial isolates using a genomic DNA extraction kit (Geneaid, USA) following the manufacturer's instructions, then the Nanodrop device was used to measure the concentration and purity of DNA by reading the absorbance at a wavelength between 260-280 nm [12].

Polymerase Chain Reaction(PCR): 40 µl of PCR mixture were prepared from DNA extraction(less or equal 250ng)from two isolats, the primers (Integrated DNA Technologies - Korea):27F : 5-AGAGTTTGATCMTGGCTCAG-3 and 1522R: 5-AAGGAGGTGATCCARCCGA-3(0.1-1.0µM) and Go teq$^{(R)}$G2Green Master Mix(1x)(Promega, USA).Supplement with nucleas free water the product to a volume of 40µl. These contents were placed in a 2.0 ml Eppendorf tube and mixed carefully. Then they put in Thermocycler according to the program as follow:

Initial denaturation performed by one cycle at 95°C for 3 mim., The second denaturation, Annealing and Extension, performed by 30 cycle at 95,58 and 72°C for 0:30,0:30,1:30 min resbectively, the final extension performed by one cycle at 72 °C for 3 min.

After the end of the program period, the product was transferred to the gel electrophoreses with 2% of agarose at 100 volts for an hour, as well as the ladder (ABM, Canada) was used. UV-trans-illuminator at 320-336nm was used to detect the bands, depending on the distance.

Sequencing of DNA: For the purpose of determining the sequence of the nitrogenous bases of *16SrRNA* gene, the PCR products of this gene were sent to psomagen sequencing Company/USA laboratory, and after obtaining the results, they were analyzed using the NCBI's BLAST program, by comparing the sequence of bases with international recorded strains.

Prodigiosin's production: Each two gram of sesame, peanut, coconut seed powder and wheat bran were added seperately to 100ml of Luria- Bertani (LB)broth medium. The pH was adjusted to 7.2, in conical flasks with a capacity of 250 ml by 50 ml per flask. Each flask of LB broth with particular material was inoculated with 2% of each bacterial isolate(S3and S4), which their concentrations were measured preliminary by absorbance at 620 nm. then they incubated in a shaker incubator at 28℃ and 200 rpm for 72 hours[13].

Extraction of prodigiosin: *S. marcescens* in Luria-Bertani broth was centrifuged at 6000 rpm for 20 minutes. The supernatant was discarded and the pellet was suspended in 95% methanol. The mixture was vortexed for 2 minutes and recentrifuged for 20 minutes at 6000 rpm. The extract was filtered by using sterile medical gauze[14].

Estimation of prodigiosin: The extracted pigment was used for the measurement of absorbance at 534 nm. prodigiosin was estimated by the following formula [15]

Prodigiosin (unit/cell) = ([OD 534 – (1.381 x OD 620)]) x 1000 / OD620

Where: OD534 = pigment absorbance; 1.381 = constant.; OD 620 = bacterial cell absorbance.

Antioxidant activity of prodigiosin: The extracted pigment was dissolved with methanol (95%) to prepare the stock solution with a concentration of 2000 µg/ml, then it was diluted with methanol to reach the required concentrations of (25, 50, 100, 200. , 400, 600, 1000 µg/ml). 1 ml of each dilution was added to 1 ml of a 0.004% methanol solution of DPPH and mixed well. After 30 min. the decrease in absorbance was measured at 517 nm. Ascorbic acid as a standard was used in the same concentrations as the pigment. The optical density was recorded for each concentration and the percentage of inhibition was calculated according to the following equation [11].

Inhibition of DPPH activity (%) = (A1 – A2) / A1x 100

Where: A1= absorbance of control ; A2= absorbance of sample

RESULTS AND DISCUSSIONS

S. marcescens identification: Two isolates (S3 and S4) carrying characteristics of *S. marcescens* were identified by characterization of red color on nutrient agar medium at 28°C, colonies appeared as convex, circular, with a smooth surface (Fig.1).These characteristics matched with mentioned previously [16].

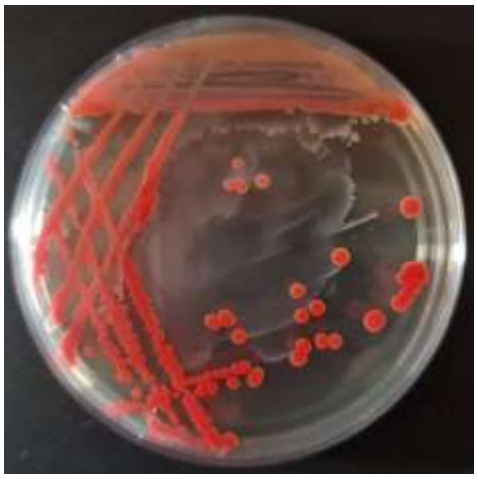

Figure 1. Growth of *S. marcescens* bacterium on nutrient agar medium at 28°C for 48 h.

DNA extracted from bacterial isolates has showed purity equal to 1.8 for S3 and 1.7 for S4 with concentrations 418.6 and 380.3 ng/µl respectively. After using PCR program, the result appeared by observing bands with a molecular size of approximately (1500bp) depending on the standard ladder used (Fig. 2).

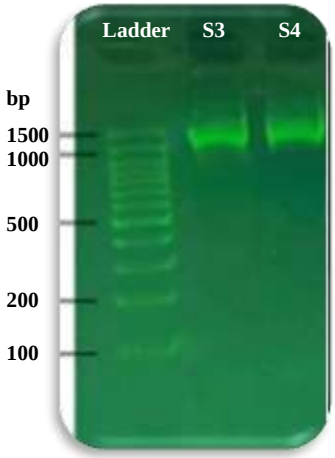

Figure 2. Electrophoresis of *16SrRNA* gene for the two *S. marcescens* isolates on an agarose gel 2% with 100 volts for an hour.

The gene sequencing revealed that the two bacterial isolates are *S. marcescens*, they came with very high similarity ratios 99.41 and 99.17% for S3 and S4 respectively with the internationally registered *S. marcescens* bacteria. These two isolates were submitted to genbank of NCBI and taken particular accession numbers, they are: OR251066 and OP251113.
Prodigiosin's production: Adding the powder of each sesame, peanut and wheat bran to Luria-Bertanii (LB) medium produced a prodigiosin pigment with a deeper color than LB medium without additives. However, the medium to which coconut was added no color was observed, therefore no prodigiosin was created (Fig. 3).

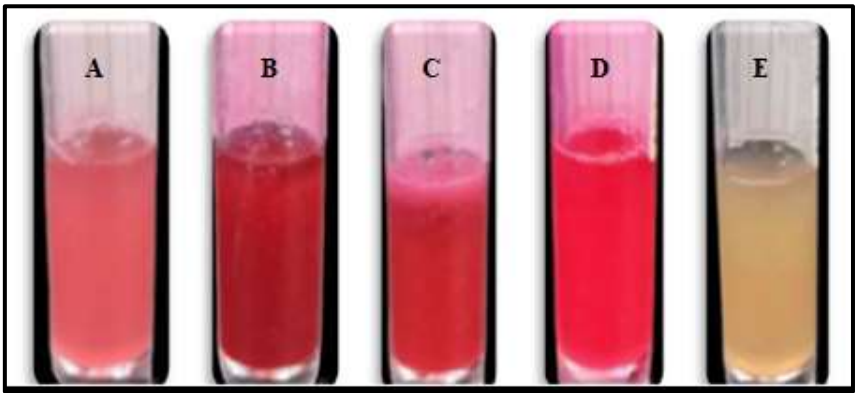

Figure 3. The color intensity of prodigiosin produced by *S. marcescens* differed according to the different natural materials added to LB broth medium after 72 h. of incubation at 28°C. A: LB ; B: LB+Sesame; C: LB+Peanut; D:LB+Wheat bran ; E: LB+Coconut

The results showed that the medium to which sesame seed powder was added gave a deeper red color than the medium supplemented with both peanut powder and wheat bran, compared to the medium of LB broth (to which no substance was added) which was characterized by its yellowish-pink color. Unfortunately the bacteria failed to produce its pigment with cocconut supplement . Sesame seed powder gave the highest concentration of the pigment, it reached 493.396 and 423.607 units/cell for S3 and S4 respectively, while wheat bran gave the lowest concentration of 400.456 units/cell for isolate S3 and 365,462 units/cell for isolate S4, while coconut powder did not record any concentration of pigment production (Table 3).

Table 3. Prodigiosin pigment concentrations of two *S. marcescens* isolates in LB broth supplemented with different materials.

Materials	**Prodigiosin concentration(unit/cell)**	
	S3	**S4**
LB	150.247	144.281
LB+Sesame	493.396	423.607
LB+Peanut	462.973	406.660
LB+Wheat bran	400.456	365.462
LB+Coconut	0	0

The increased production of prodigiosin pigment with the addition of natural powders is due to the fact that the seeds are composed of minerals, vitamins and saturated and unsaturated fatty acids and these constituents differ from seed to seed[17], as *S. marcescens* is capable of producing lipase enzyme, which in turn degrades these fatty acids for use by bacteria as a carbon source [18].

The success of pigment production in the medium to which wheat bran powder was added may be due to the fact that wheat bran is a material rich in fiber, minerals and vitamins, with a lower percentage of fat [19], but in our result gave the lowest amount of pigment. These results are consistent with what was mentioned in previous studies[20,21].The reason for not producing prodigiosin in coconut powder medium could be inorder to contain 50% capric acid and 70% Lauric acid, which have an inhibitory effect against bacteria, and thus is reflected in the metabolism of the pigment[22].

Applying the prodigiosin as antioxidant: The addition of prodigiosin pigment to DPPH caused color changing from dark purple to yellow and white. It can be noted that, the antioxidant activity of prodigiosin increased gradually with increasing concentration(Fig.4),compared to standard ascorbic acid ,our result indicates that the antioxidant activity of prodigiosin is little lower than acid. (Fig.5).

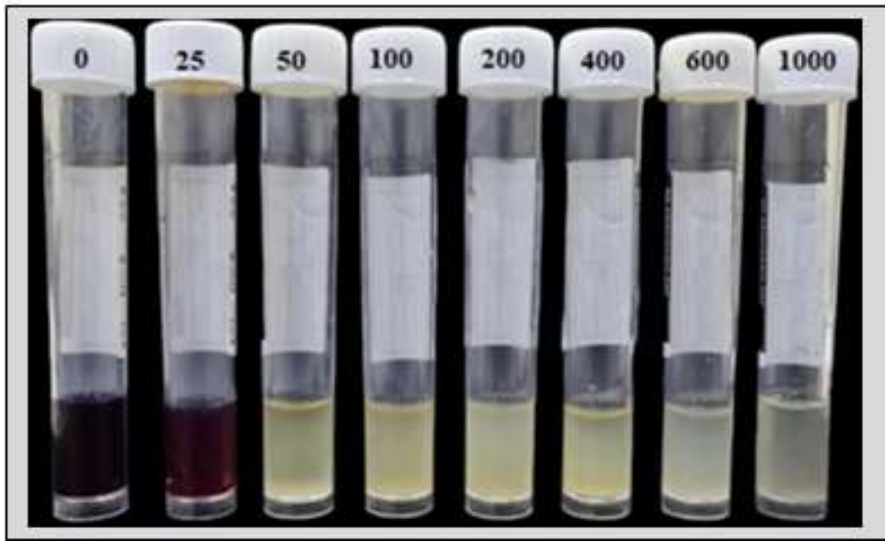

Figure 4. Changing the color intensity of the DPPH solution by increasing prodigiosin's concentration

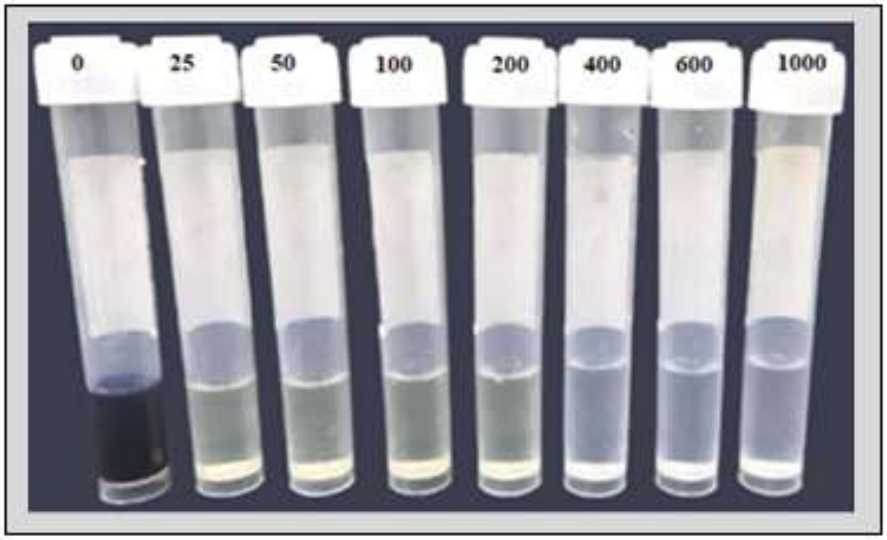

Figure 5. Changing the color intensity of the DPPH solution by increasing ascorbic acid's concentration

From Table (4) the antioxidant activity of prodigiosin increased gradually with increasing concentration. The DPPH free radicals scavenging activity of prodigiosin were found to be 67, 73 , 75, 77, 79, 97 and 97% at 25, 50, 100, 200, 400, 600 and 1000 µg/ml of prodigiosin respectively. This result agrees with what was mentioned in previous studies[23,24,11].

Table 4. Antioxidant of prodigiosin

Prodigiosin concentration(µg/ml)	**Prodigiosin's antioxidant activity (%)**	**Ascorbic acid's antioxidant activity (%)**
25	67	96
50	73	97
100	75	97
200	77	97
400	79	99
600	97	100
1000	97	100

Antioxidants donate protons to free radicals to neutralize the charge in solution, and then reduce the intensity of absorption at a wavelength of 517 nm[25]. The strong activity of prodigiosin as an antioxidant is attributed to the structures of the double bond and the pyrrole ring of prodigiosin where the protonated hydrogen of the pyrrole ring C in prodigiosin interacts with the N˙ radical of DPPH molecule, causing the color of DPPH to change from dark purple to yellow and sometimes white[23,24]. The result of the prodigiosin ability to oxidize DPPH was identical to the results of previous, where one study showed that the use of the prodigiosin pigment at 600 µg/ml gave removal of free radicals for the DPPH

molecule amounted to 95[26], and in another study it was found that the removal rate of free radicals reached 92% when using the pigment at a concentration of 100% [11].

CONCLUSION

The study succeeded in producing a high yield of prodigiosin pigment from local *S. marcescens* isolates induced by available natural, cheap materials. Results showed that sesame seed powder was the best source of prodigiosin, followed by peanut seeds and wheat bran. prodigiosin showed antioxidant activities, so it can be used in the food and pharmaceutical industries.

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